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A Comparative Study of Phytochemical Constituents and Bioactivity of *n*-Hexane and dichloromethane extracts of *Juniperus oxycedrus* subsp. *macrocarpa* and *J. oxycedrus* subsp. *Oxycedrus* †

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Abstract: This study assessed and compared the chemical profile and the antioxidant and anti-proliferative activities of non polar extracts of *Juniperus oxycedrus* subsp. *macrocarpa* (Sibth. & Sm.) Ball. and *J. oxycedrus* subsp. *oxycedrus* L. from Italy. The aerial parts of both *Juniperus* subspecies were subjected to exhaustive macerations with *n*-hexane and dichloromethane as solvents. Extracts were investigated for their chemical profile by gas chromatography (CG) and gas chromatography-mass spectrometry (GC-MS). Ferric Reducing Activity Power (FRAP), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH), and β-carotene bleaching assays were applied to study the antioxidant properties. The cytotoxic activity was evaluated using the sulforhodamine B (SRB) assay against several cancer human cell lines (MCF-7, MDA-MB-231, A549, and COR-L23 cells). Monoterpenes and sesquiterpenes mainly characterized *n*-hexane extracts while diterpenes and fatty acids are the most abundant identified compounds in dichloromethane extracts. The *n*-hexane and dichloromethane extracts of *J. oxycedrus* subsp. *oxycedrus* showed the most promising cytotoxic activity against COR-L23 cell line with IC_{50} values of 26.9 and 39.3 μg/mL, respectively. *J. oxycedrus* subsp. *macrocarpa* revealed a great radicals scavenging activity. Overall, obtained results suggest both *Juniperus* subspecies as good source of potential antioxidants and anti-proliferative compounds.

Keywords: *Juniperus*; phytochemical profile; antioxidant; anti-proliferative activity

1. Introduction

The genus *Juniperus* (Cupressaceae) includes about 60 species native plants of the Mediterranean regions. *Juniperus oxycedrus* L. is a shrub or small tree native across the Mediterranean region from Morocco and Portugal east to western Caucasus, growing on a variety of rocky sites from sea level up to 1600 m altitude [1]. According to the Flora Europea, *J. oxycedrus* L. includes three subspecies: subsp. *oxycedrus*, subsp. *macrocarpa* (Sibth. & Sm.) Ball. and subsp. *badia* (H. Gay) Debeaux.

Many biological effects exerted by *Juniperus* species, including antioxidant, antimicrobial, antiviral, anticancer, and antifungal activities, are reported in literature [2,3].

Cancer is one of the major causes of mortality in the world and due to its prevalence the discovery of novel anticancer agents is of great importance [4].

Phytochemicals and their derivatives have showed a remarkable potential for the development of chemotherapeutic agents. Exemples are paclitaxel, vinblastine, vincristine, used for the treatment of several types of cancer.

The increased production of reactive oxygen species (ROS) and/or the decrease of the antioxidant defense systems are responsible for oxidative stress [5]. Several works have suggested the implication of ROS in the underlying molecular mechanisms involved in initiation, promotion and progression of carcinogenesis and proposed antioxidants are an important line of defense to regulate significant signaling transduction pathways, including nuclear factor kB, mitogen-activated protein kinases, nuclear factor erythroid-2-related factor 2, and phosphatidylinositide 3-kinases/protein kinase B, by repairing damaged DNA, reducing cell proliferation, angiogenesis, and, metastasis. Therefore, cancer cells can be prevented and reversed without harming normal cells by oxidative modifications of DNA, leading to the reduction of ROS levels.

In this context, herein we have investigated the chemical profile, in vitro antioxidant effects, and anti-proliferative activities against four human cancer cell lines of *n*-hexane and dichloromethane extracts of *Juniperus oxycedrus* subsp. *macrocarpa* and *J. oxycedrus* subsp. *oxycedrus* collected in Southern Italy.

2. Materials and Methods

2.1. Plant Materials and Extraction Procedure

The aerial parts of *Juniperus oxycedrus* subsp. *macrocarpa* were harvested in Isola di Capo Rizzuto (Sounther Italy). The aerial parts of *J. oxycedrus* subsp. *oxycedrus* were harvested in Corigliano Calabro (Cosenza, Sounther Italy). Samples were authenticated by Dr. NG Passalacqua at the Natural History Museum of Calabria and Botanic Garden, University of Calabria (Rende, CS, Italy).

The aerial parts of *Juniperus oxycedrus* subsp. *macrocarpa* (200 g) and *J. oxycedrus* subsp. *oxycedrus* (200 g) were extracted by maceration method using *n*-hexane (1L) and dichloromethane (1 L) as sovents. The extraction procedure was repeated three times and each process maintained 72 h. Yields of 0.6 and 0.4% for *n*-hexane extracts, and 3.5 and 4.0% for dichloromethane extracts of *Juniperus oxycedrus* subsp. *macrocarpa* and *J. oxycedrus* subsp. *oxycedrus*, respectively, were obtained.

2.2. Chemical Analyses

Chemical analyses were performed by gas chromatography-mass spectrometry (GC-MS) and gas chromatography (GC) [6]. In brief, GC-MS analyses were carried out on a Hewlett-Packard 6890 gas chromatograph with a fused silica HP-5 capillary column (30 m length, 0.25 mm i.d., 0.25 μm film thickness) and helium as carrier gas (Agilent, Milan, Italy).

Ionization energy voltage 70 eV was used. Column temperature was initially kept at 50 °C for 5 min, then increased to 280 °C at 13 °C/min, held for 10 min at 280 °C.

GC analyses were performed using a Shimadzu GC17A gas chromatograph (Shimadzu, Milan, Italy) equipped with flame ionization detector (FID) and using a HP-5 MS capillary column (30 m × 0.25 mm i.d.; film thickness 0.25 μm) (Agilent, Milan, Italy). Flame ionization detection (FID) was performed at 280 °C. Nitrogen was the carrier gas. The temperatures were programmed as described above. Constituents were tentatively identified comparing their retention times either with those in the literature or with those of authentic compounds available in our laboratory. Further identification was made by comparing their mass spectra with either those stored in Wiley 275 library or with mass spectra from the literature and from our in-house library [7].

2.3. In Vitro Anti-Proliferative Activity

2.3.1. Cells Culture

Four human cancer cell lines, such as breast cancer MCF-7, triple negative breast adenocarcinoma MDA-MB-231, lung large cell carcinoma COR-L23, and lung carcinoma A549, purchased from the ATCC from the American Type Culture Collection (ATCC) (Manassas, VA), were used. COR-L23 cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium, while A549, MCF-7, and MDA-MB-231 cells were maintained in Dulbecco's modified Eagle's medium (DMEM). Both media were supplemented with 10% fetal bovine serum, 1% L-glutamine, and 1% penicillin/streptomycin. Cell lines were maintained at 37 °C in a 5% CO_2 atmosphere with 95% humidity.

2.3.2. Sulforhodamine B (SRB) Assay

The potential anti-proliferative activity of *J. oxycedrus* subsp. *macrocarpa* and *J. oxycedrus* subsp. *oxycedrus* extracts was assessed by using the sulforhodamine B (SRB) assay [8].

In this assay, cells were placed in 96-well plates in a range from 5×10^4 to 15×10^4 cells. After 24 h, cells were treated with 100 mL/well of different concentrations of extracts. After 48 h, cells were fixed with ice-cold 40% trichloroacetic acid, and were stained with 50 mL of 0.4 % (w/v) SRB in 1% acetic acid. After 30 min, the plates were washed with 1% acetic acid and air-dried overnight. For reading plates, the bound dye was solubilised with 100 mL of 10 mM tris(hydroxymethyl)aminomethane (Tris base). The absorbance was read at 490 nm (Molecular Devices SpectraMax Plus Plate Reader, Celbio, Milan, Italy). Cell survival was measured as percentage absorbance compared with the untreated control.

2.4. Antioxidant Tests

The antioxidant activity of *J. oxycedrus* subsp. *macrocarpa* and *J. oxycedrus* subsp. *oxycedrus* extracts were investigated by using a) Ferric Reducing Antioxidant Power (FRAP), b) 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic) acid (ABTS), c) 2,2-diphenyl-1-picrylhydrazyl (DPPH), and d) β-carotene bleaching tests.

For the preparation of FRAP reagent, a mixture of tripyridyltriazine (TPTZ) solution, HCl, $FeCl_3$, and acetate buffer (pH 3.6) was prepared as reported by Tenuta et al. [9]. Extracts at a concentration of 2.5 mg/mL in ethanol were mixed with FRAP reagent and water. After 30 min of incubation at 25 °C, the absorption was measured at 595 nm.

ABTS assay was applied as previously described [9]. A solution of ABTS radical cation was prepared. After 12 h, this solution was diluted with ethanol to an absorbance of 0.70 at 734 nm using a UV-Vis Jenway 6003 spectrophotometer. Dilution of extracts in ethanol were added to 2 mL of diluted ABTS solution in order to test concentrations in the range 400–1 μg/mL. After 6 min, the absorbance was read at 734 nm.

DPPH radical scavenging activity was determined according to the technique previously reported [9]. An aliquot of 1.5 mL of 0.25 mM DPPH radical (DPPH·) in ethanol was mixed with 12 μL of samples in order to test concentrations ranging from 1000 to 1 μg/mL. The absorbance was determined at 517 nm with a UV-Vis Jenway 6003 spectrophotometer. In the β-carotene bleaching test, a mixture of linoleic acid, Tween 20, and β-carotene was prepared as previously described [9].

β-Carotene was added to linoleic acid 100% Tween 20. After evaporation of the solvent and dilution with water, the emulsion was added to a 96-well microplate containing samples in ethanol concentrations ranging from 100 to 2.5 μg/mL. The plate was left to incubate at 45 °C for 30 and 60 min. The absorbance was measured at 470 nm.

2.5. Statistical Analysis

Data are expressed as means ± standard deviation (S.D.) (n= 3). IC_{50} values were calculated by using Prism GraphPad Prism version 4.0 for Windows (GraphPad Software, San Diego, CA, USA).

Results were statistically analyzed by using one-way analysis of variance test (ANOVA) followed by a multicomparison Dunnett's test (α = 0.05).

3. Results

3.1. Chemical Profile

The non polar extracts of J. oxycedrus subsp. macrocarpa and J. oxycedrus subsp. oxycedrus were analyzed by gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS).

The n-hexane extract of J. oxycedrus subsp. macrocarpa showed α-pinene, p-cymene, manoyl oxide, 13-epi-manoyl oxide, α-terpeneol, α-cubebene, β-cubebene, lauric acid, palmitic acid, ethyl palmitate, ethyl linoleate, and ethyl linolenate as main constituents.

The n-hexane extract of J. oxycedrus subsp. oxycedrus is characterized by the presence, as most abundant constituents, of verbenone, α-cubebene, 1-octadecene, abieta-8,11,13-trien-7-one, cadalene, ferruginol and different fatty acids, such as lauric acid, ethyl laurate, palmitic acid, ethyl palmitate, ethyl myristate, stearctic acid, methyl lignocerate.

α-Pinene, myrcene, p-cymene, manoil oxide, γ-cadinene, δ-cadinene, sandaracopimaradiene, abietatriene, abietadiene, dehydroabietal, and several alkanes are the main constituents identified in the dichloromethane extract of J. oxycedrus subsp. macrocarpa.

On the other hand, the dichloromethane extract of J. oxycedrus subsp. oxycedrus showed as more abundant constituents, the abietane diterpenes abietatriene, abietadiene, and ferruginol, the coumarin umbelliferone, some fatty acids and their derivatives (palmitic acid, ethyl palmitate, ethyl myristate, stearctic acid, and methyl lignocerate).

3.2. Anti-Proliferative Activity

In order to investigate the effects on cellular viability of the aerial parts of *J. oxycedrus* subsp. *macrocarpa* and *J. oxycedrus* subsp. *oxycedrus*, extracts were investigated by using SRB assay against four human cancer cell lines, namely MCF-7, MDA-MB-231, A549, and MDA-MB-231 cells (Table 1).

Control experiments using non-tumorigenic 3T3-L1 cells were performed.

Table 1. Anti-proliferative activity (IC_{50} μg/mL) of *J. oxycedrus* subsp. *macrocarpa* and subsp. *oxycedrus*.

	MCF7	MDA-MB-231	A549	COR-L23	3T3-L1
J. oxycedrus subsp. *macrocarpa*					
n-Hexane	>200	>200	>200	75.1 ± 2.5 **	>200
Dichloromethane	>200	>200	>200	>200	>200
J. oxycedrus subsp. *oxycedrus*					
n-Hexane	>200	>200	46.0 ± 2.4 **	26.4 ± 1.3 **	>200
Dichloromethane	103.8 ± 3.8 **	126.8 ± 4.8 **	59.7 ± 1.9 **	39.3 ± 1.8 **	>200
Positive control					
Taxol	0.08 ± 0.004	1.61 ± 0.03			
Vinblastin			67.3 ± 2.0	45.5 ± 0.7	43.8 ± 0.9

Data are expressed as mean ± standard deviation (SD) ($n = 3$). ** $p < 0.001$ vs. control.

Both *n*-hexane and dichloromethane extracts of *J. oxycedrus* subsp. *oxycedrus* showed a promising cytotoxic activity against COR-L23 cell line with IC_{50} values of 26.9 and 39.3 μg/mL, respectively. The same extracts are able also to inhibit the lung carcinoma (A549) cell growth with IC_{50} values of 46.0 and 59.3 μg/mL, for *n*-hexane and dichloromethane, respectively.

J. oxycedrus subsp. *macrocarpa* extracts were not active at the highest tested concentration, except for the *n*-hexane extract against COR-L23 (IC_{50} value of 75.1 μg/mL). None of the tested extracts affected the proliferation of 3T3-L1 cells suggesting a selective action against cancer cells.

3.3. Antioxidant Activity

The antioxidant properties of *Juniperus* subspecies were studied by four methods, namely DPPH, ABTS, FRAP, and ββ-carotene bleaching assays. Results are summarised in Table 2.

Table 2. In vitro antioxidant activity of *J. oxycedrus* subsp. *macrocarpa* and *J. oxycedrus* subsp. *oxycedrus* extracts.

Sample	ABTS IC_{50} (μg/mL)	DPPH IC_{50} (μg/mL)	FRAP Test μM Fe(II)/g[c]	β-Carotene Bleaching Test IC_{50} (μg/mL)	
				30 min	60 min
Juniperus oxycedrus subsp. *macrocarpa*					
n-Hexane	4.0% [a]	48.8 ± 2.8 ****	6.4 ± 0.3 ****	25.1%[d]	12.5%[d]
Dichloromethane	16.8 ± 1.1 ****	44.8 ± 2.6****	11.7 ± 1.2 ****	71.4 ± 2.2 ****	90.9 ± 1.2 ****
Juniperus oxycedrus subsp. *oxycedrus*					
n-Hexane	19.4%[a]	31.9% [b]	6.9 ± 0.9 ****	22.07%[d]	25.40%[d]
Dichloromethane	73.2 ± 2.7 ****	131.9 ± 5.3 ****	34.9 ± 1.9 ****	26.4 ± 1.8 ****	87.0 ± 3.8 ****
Positive control					
Ascorbic acid	1.7 ± 0.4	5.1 ± 0.8			
BHT			63.2 ± 4.4		
Propyl gallate				1.1 ± 0.05	1.0 ± 0.06

Data are expressed as means ± S.D. (n= 3). Antioxidant Capacity Determined by Radical Cation (ABTS+); DPPH Radical Scavenging Activity Assay; Ferric Reducing Antioxidant Power (FRAP); β-carotene bleaching test; Ascorbic acid, BHT and Propyl gallate were used as positive control in antioxidant tests. Differences within and between groups were evaluated by one-way ANOVA followed by a multicomparison Dunnett's test ($\alpha = 0.05$): **** $p < 0.0001$ compared with the positive controls.

Dichloromethane extracts of both *Juniperus* subspecies exhibited the highest activity. Considering the radicals scavenging activity, the dichloromethane extract of *Juniperus oxycedrus* subsp. *macrocarpa* was the most active with IC_{50} values of 16.8 and 48.8 μg/mL in ABTS and DPPH tests, respectively. Conversely, the dichloromethane extract of *J. oxycedrus* subsp. *oxycedrus* was the most active in FRAP and β-carotene bleaching tests with a value of 34.9 μM Fe(II)/g in FRAP test and IC_{50} values of 26.4 and 87.0 μg/mL after 30 and 60 min of incubation, respectively, in the β-carotene bleaching test.

4. Discussion

An increased number of studies demonstrated the importance of *Juniperus* species as source of phytochemicals with promising anti-cancer effects [2,3]. In the current work, the anti-proliferative activity of non polar extracts of two *Juniperus* species have been investigated. *J. oxycedrus* subsp. *oxycedrus* showed greater and more promising anti-proliferative activity against lung carcinoma than *J. oxycedrus* subsp. *macrocarpa*. These data are better than those obtained with the positive control vinblastin. Instead, except for the *n*-hexane extract, *J. oxycedrus* subsp. *macrocarpa* exhibited a good radicals scavenging activity in both ABTS and DPPH tests.

Some abietane diterpenes have been identified in the dichloromethane extract of *J. oxycedrus* subsp. *oxycedrus*. In the literature survey, the abietane diterpene ferruginol demonstrated to possess anti-cancer [10,11]. Jia et al. [10] demonstrated that ferruginol suppressed the proliferation of SK-Mel-28 human malignant melanoma cells in a concentration-dependent and time-dependent manner through induction of apoptosis. Apoptotic effects was mediated via p38 phosphorylation and RELA translocation to nucleus.

Another compound identified in *J. oxycedrus* subsp. *oxycedrus* dichloromethane extract is umbelliferone. This coumarin has been reported to exhibit in vivo anti-tumor and immunomodulatory effects against sarcoma 180, [12]. Moreover, Yu et al. [13] reported the anti-cancer activity in human hepatoma cells HepG2 of umbelliferone through the induction of apoptosis and cell cycle arrest. Umbelliferone inhibited proliferation and migration of laryngeal cancer cells [14]. The anti-cancer potential effects of umbelliferone have also been described against 7,12-dimethylbenz(a)anthracene-induced rat mammary carcinoma [15].

Previous studies have reported cytotoxic effects of some *Juniperus* species on several human cancer cell lines including lung cancer cells A549 [2]. Different extracts have been tested. Among them, the methanol extracts of *J. oxycedrus* subsp. *oxycedrus* and *J. oxycedrus* subsp. *macrocarpa* from Turkey were assessed against the human hepatocellular liver carcinoma (HepG2 cells). However, no effects on viability of these cell line were reported [16]. In another work, De Marino et al. [17] investigated the potential activity of a *n*-butanol extract of *J. oxycedrus* subsp. *oxycedrus* against breast cancer (MCF-7 cell line), malignant melanoma (A375 cell line) and lung carcinoma (H460 cell line). Interesting results were obtained only against MCF-7 cancer cells.

5. Conclusions

This work was designed to make a comparative study of *n*-hexane and dichloromethane extracts of two *Juniperus* subspecies namely *J. oxycedrus* subsp. *macrocarpa* and *J. oxycedrus* subsp. *oxycedrus* as potential source of antioxidant and anti-proliferative agents.

In order to investigate the cytotoxic effects of the *n*-hexane and dichloromethane extracts of *Juniperus* subspecies on cancer cells, four human cancer cell lines (MCF-7, MDA-MB-231, A549, and COR-L23) were selected. The analysis of obtained results showed a promising and selective anti-proliferative activity of both extracts of *J. oxycedrus* subsp. *oxycedrus* in a concentration-dependent manner against lung cancer cells lines A549 and COR-L23. *J. oxycedrus* subsp. *oxycedrus* may represents the best candidate for further in vivo studies to find new natural potential anticancer compounds. Our results confirm the prominent role of plant-derived molecules in the search for new therapeutic compounds.

Author Contributions: M.B. carried out the overall project design experimental work; M.L. and R.T. analysed results; R.T., M.R.L. and M.B. wrote the manuscript; R.T. and M.R.L. conceptualized aspects of the project and assisted with reviewing and editing the manuscript. All authors read and agree to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

The following abbreviations are used in this manuscript:

ABTS	2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic) acid
BHT	Butylated hydroxytoluene
DPPH	2,2-diphenyl-1-picrylhydrazyl
FRAP	Ferric Reducing Antioxidant Power
IC_{50}	half maximal inhibitory concentration
ROS	Reactive Oxygen Species
SD	Standard Deviation
SRB	Sulforhodamine B
TPTZ	tripyridyltriazine

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